De Novo Biosynthesis of Fatty Acids Plays Critical Roles in the Response of the Photosynthetic Machinery to Low Temperature in Arabidopsis

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The Arabidopsis thaliana kas3 mutant was isolated based on the hypersensitivity of PSII to low temperature using a Chl fluorescence imaging technique. Chl content was lower in kas3 seedlings cultured at 23°C than in the wild type, but PSII activity was only mildly affected. However, after the chilling treatment at 4°C for 7 d, PSII activity was severely impaired in kas3. PSII was more sensitive to light at 4°C in the presence of lincomycin, suggesting that the kas3 mutation accelerates at least the PSII photodamage. The kas3 mutation causes an amino acid alteration in 3-ketoacyl-ACP synthase III (KasIII), leading to the partial loss of the de novo synthesis pathway for fatty acids in plastids. Consequently, the total fatty acid level was reduced to 75% of the wild-type level in kas3 at 23°C and was further reduced to 60% at 4°C. The composition of fatty acids was also slightly affected in kas3 at both 4 and 23°C. Consistent with the results of the electron transport analysis, the chilling treatment also destabilized PsaA and cytochrome (Cyt) f and D1 in kas3. An analysis of double mutants with pgr1 conditionally defective in Cyt b6f activity and with var2 defective in FtsH protease suggested that the kas3 mutation has pleiotropic effects on chloroplast function, probably impacting both the Cyt b6f activity and translation in chloroplasts at 23°C. The full activity of KasIII is required for the biogenesis of the intact electron transport machinery in thylakoid membranes and is especially important for the process of responding to low temperature.

Keywords: Arabidopsis • Chloroplast • Fatty acid • Low temperature • Photosynthesis • PSII.

Introduction

PSII is the site of light-driven water:plastoquinone oxidoreduction in oxygenic photosynthesis, and it is the most light-sensitive part of the electron transport machinery in thylakoid membranes (Aro et al. 1993). Consequently, PSII is often photo-inhibited, especially under stress conditions (Murata et al. 2007). Photoinhibition results from an imbalance between the rate of PSII photodamage and its repair. A recent model suggests that PSII photodamage is proportional to light intensity, while environmental stresses impair the PSII repair cycle, resulting in accelerated PSII photoinhibition (Tyystjärvi and Aro 1996, Nishiyama et al. 2001, Takahashi and Murata 2008). The PSII repair cycle involves a series of processes (Aro et al. 2005). Damaged PSII migrates from the grana thylakoids to the stroma lamellae, where the PSII core is partially disassembled. Then, the damaged D1 protein is specifically removed by proteolysis and replaced by the de novo synthesized precursor D1. Finally, the precursor D1 is processed into the mature form to restore PSII activity.

At low temperatures, photosynthesis is inhibited in the presence of light (Powles 1984). Plants counteract this low temperature photo-inhibition in multiple ways, and one of their main strategies is to regulate membrane fluidity by modifying the unsaturation levels of lipids (Iba 2002). The saturation levels of phosphatidylglycerol (PG) correlate well with chilling sensitivity (Murata et al. 1982, Murata 1983). Phenotypes of Arabidopsis mutants (Hugly and Somerville 1992, Miquel et al. 1993) and transgenic tobacco expressing squash or cyanobacterial genes (Murata et al. 1992, Ishizaki-Nishizawa et al. 1996) support this idea. The same strategy is also used by plants to resist high temperature (Murakami et al. 2000). However, the isolated thylakoids from transgenic tobacco that accumulates less unsaturated fatty acids did not show significant sensitivity to low temperature (Moon et al. 1995). In contrast, these transgenic plants recovered from PSII photoinhibition more slowly.
than the wild-type plants, suggesting that unsaturation of fatty acids, especially of PG, is required for the efficient PSII repair cycle at low temperatures. This result is consistent with the idea that environmental stresses inhibit the repair of damaged PSII, leading to enhanced PSII photoinhibition.

Thylakoid membranes are mainly composed of galactolipids, such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) (Wada and Murata 2007). PG is also included in thylakoid membranes and its level varies with the phosphate status (Yu et al. 2002). In addition to their roles as membrane constituents, some lipid molecules have been identified in crystal structures of the electron transport machinery. The PSI complex of *Thermosynechococcus elongatus* contains one molecule of MGDG and three molecules of PG (Jordan et al. 2001). They are suggested to be involved in the formation of the reaction center, the trimerization of PSI and the binding of Psax to the PSI complex. Compared with PSI, the PSII complex of *T. elongatus* contains more lipids: 11 molecules of MGDG, seven of DGDG, five of SQDG and two of PG (Guskov et al. 2009). Of these 25 lipid molecules, 18 are located close to the reaction center, probably lending it structural flexibility. This flexibility may be required for the replacement of damaged D1 in the PSII repair cycle (Kanervo et al. 1995). One molecule of PG and one molecule of DGDG were also found in light-harvesting complex II (LHClI), and they are probably involved in trimer formation and the interactions between trimers, respectively (Liu et al. 2004). The Cyt b6f complex was also shown to contain two phosphatidylincholine molecules in *Mastigocladus laminosus* (Kurisu et al. 2003) and one SQDG molecule and two additional lipid molecules in *Chlamydomonas reinhardtii* (Stroebel et al. 2003).

In this study, we characterized an Arabidopsis thaliana mutant (*kas3*) whose PSII is sensitive to low temperature. The *kas3* mutant is partially defective in the de novo synthesis pathway of fatty acids in plastids and exhibits pleiotropic phenotypes in chloroplast function. The *kas3* mutation has pleiotropic effects on the function of the electron transport machinery, including accelerating PSII photodamage at low temperature. We discuss the function of de novo fatty acid biosynthesis during the protective responses to low temperature.

### Results

#### PSII is hypersensitive to low temperature in *kas3*

To study the molecular mechanisms that maintain the photosynthetic machinery at low temperatures, we screened Arabidopsis mutants exhibiting PSII photoinhibition under chilling conditions (4°C for 7 d under continuous light) using a Chl fluorescence imaging system. The Chl fluorescence parameter $F_v/F_m$ represents the maximum activity of PSII and can be used to monitor PSII photoinhibition. In the wild type, $F_v/F_m$ was only slightly lower after chilling treatment than before the treatment (Table 1), indicating that this stress was not very deleterious. In contrast, $F_v/F_m$ was significantly lower after the chilling treatment in the *kas3* mutant (Table 1), which is named after the enzyme responsible for the phenotype, KasIII, for 3-ketoacyl-ACP synthase III. Before the stress, $F_v/F_m$ was also slightly lower in *kas3* than in the wild type (Table 1). Even before the chilling treatment, the leaves of *kas3* plants were pale-green (Fig. 1) and Chl content was reduced (Table 1), although plant growth was only slightly affected.

To characterize the *kas3* photosynthetic electron transport phenotype in more detail, the light intensity dependence of the electron transport rate (ETR) and non-photochemical quenching of Chl fluorescence (NPQ) was compared between *kas3* and wild-type plants before and after low temperature treatment (Fig. 2A, B). While the ETR reflects the relative rate of electron transport through PSII during steady-state photosynthesis, NPQ is mainly caused by thermal dissipation of absorbed excess light energy in flowering plants, a process that depends on a build-up of ΔpH across the thylakoid membrane (Krause and Weis 1991). At 23°C, neither the ETR nor NPQ was significantly affected in *kas3* (Fig. 2A, B), consistent with their growth phenotype (Fig. 1A). After the chilling treatment, the ETR was saturated at a lower level and higher NPQ was induced at lower light intensities in the wild type. This result suggests that plants adapted to the chilling conditions by using less light energy for photosynthesis. In *kas3*, the ETR level was drastically low, probably leading to lower NPQ induction (Fig. 2A, B). This is consistent with the fact that PSII is severely photodamaged by low temperatures in *kas3* as evidenced by a decline in $F_v/F_m$.

To characterize further the defect in photosynthetic electron transport in *kas3*, the redox levels of PSI were determined under the growth conditions used and after chilling treatment (Fig. 2C). The parameter $\Delta A/\Delta A_{\text{max}}$ indicates the oxidation state of the special pair of reaction center Chls in PSI (P700). In the wild type, P700 was oxidized more with an increase in light intensity, probably due to the down-regulation of PSI photochemistry by NPQ and the restriction of electron transport at the Cyt b6f complex. Despite the defect in NPQ induction in *kas3* after chilling treatment (Fig. 2B), P700 was more oxidized in *kas3* than in the wild type (Fig. 2C).

<table>
<thead>
<tr>
<th>Table 1 Total Chl content and $F_v/F_m$</th>
<th>Wild type</th>
<th><em>kas3</em></th>
<th><em>kas3 + KasIII</em></th>
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<tr>
<td>Total Chl (µg cm$^{-2}$)</td>
<td></td>
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<tr>
<td>23°C</td>
<td>12.4 ± 0.9</td>
<td>9.0 ± 0.9</td>
<td>11.1 ± 0.9</td>
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<tr>
<td>4°C</td>
<td>13.7 ± 0.6</td>
<td>8.4 ± 1.0</td>
<td>12.8 ± 0.6</td>
</tr>
<tr>
<td>$F_v/F_m$</td>
<td></td>
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<tr>
<td>23°C</td>
<td>0.784 ± 0.007</td>
<td>0.712 ± 0.019</td>
<td>0.784 ± 0.005</td>
</tr>
<tr>
<td>4°C</td>
<td>0.744 ± 0.022</td>
<td>0.323 ± 0.085</td>
<td>0.747 ± 0.023</td>
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After chilling treatment (continuous light of 50 µmol photons m$^{-2}$ s$^{-1}$, 4°C for 7 d), plants were dark adapted for 1 h at room temperature before the $F_v/F_m$ measurement. Means ± SD (n = 6).
before P700 in kas3 than in the wild type after chilling treatment.

Taking all the results of electron transport analysis together (Fig. 2), chilling treatment affects photosynthetic electron transport at least in PSII and also the inter-system between PSII and P700. It is probable that the kas3 defect pleiotropically affects the function of the photosynthetic machinery in the thylakoid membranes.

**kas3 is defective in thylakoid protein accumulation at low temperature**

In kas3, photosynthetic electron transport is hypersensitive to low temperature (Fig. 2). It is possible that the kas3 defect affects the accumulation of the electron transport machinery in thylakoid membranes at low temperatures. To test this possibility, levels of plastid-encoded D1 (PSII), PsaA (PSI) and Cyt f (Cyt b6f complex) and nucleus-encoded PsbO (PSII oxygen-evolving complex) were compared between wild-type and kas3 plants before and after chilling treatment (Fig. 3A). As a control, the level of BiP (a molecular chaperone in the endoplasmic reticulum) was also determined. At 23°C, the levels of PsaA and Cyt f were comparable between the wild type and kas3, but D1 levels were reduced by less than a half in kas3 when normalized on the basis of Chl. Since the Chl level in kas3 is 73% of that found in the wild type (Table 1), kas3 contains much less D1 per leaf area. The slightly lower $F_v/F_m$ in kas3 than in the wild type at 23°C (Table 1) could be explained by the instability of PSII. We conclude that the accumulation and activity of PSII is partially impaired even at 23°C in kas3.

In the wild type the chilling treatment decreased the D1 level to 25–50% of that before the stress (Fig. 3A). In contrast, the level of Cyt f appeared slightly higher after the chilling treatment in the wild type. The D1 level also decreased in kas3 after the chilling treatment, and this was accompanied by a drastic loss of PSII activity (Table 1). In contrast to the drastic reduction in D1 level, the PsbO level was only slightly affected in kas3 after the chilling treatment. The Cyt f level was reduced after the stress to approximately 50% of that before it (Fig. 3A). This result is consistent with the fact that electron transport is restricted in the inter-system in kas3 at the chilling temperature (Fig. 2C). Although the PsaA level was constant before and after the chilling treatment in the wild type, it was reduced after the stress in kas3. Consistent with the results of electron transport analysis, the kas3 defect pleiotropically affected the stability of PSII, PSI and the Cyt b6f complexes. In contrast to chloroplast proteins, the BiP level was higher in kas3 both before and after the chilling treatment than in the wild type (Fig. 3A). As the samples were loaded for SDS–PAGE on the basis of Chl level, which was lower in kas3, the BiP level is unlikely to be affected in kas3.

**Fig. 1** Detection of chilling sensitivity by Chl fluorescence imaging. (A) Wild-type (WT) and kas3 seedlings before and after chilling treatment (continuous light of 50 ìmol photons m$^{-2}$ s$^{-1}$ at 4°C for 1 week). (B) Chl fluorescence images of the upper panel. Images represent the maximum activity of PSII ($F_v/F_m$).
The kas3 phenotype is caused by a single amino acid alteration in KasIII

To identify the gene affected in kas3 (Columbia gl1), the mutant was crossed with the polymorphic wild type (Landsberg erecta). Analysis of 277 F₂ plants and 319 F₃ plants identified a 161 kb region flanked by two molecular markers, F24O1 and F23N19 (Fig. 4A). As the kas3 phenotype was observed mainly in the chloroplast function, genes encoding possible plastid targeting signals (predicted by TargetP. http://www.cbs.dtu.dk/services/TargetP/) were sequenced. Finally, a single mutation was identified in a gene, At1g62640. The well-conserved 196th glycine (GGC) was substituted by serine (AGC) in At1g62640 of kas3 (Fig. 4B).

To verify that the kas3 phenotype is caused by the mutation in At1g62640, a genomic fragment containing only the complete At1g62640 gene was introduced into kas3. The transformation fully restored the Fv/Fm value after the chilling treatment, Chl content at 23°C (Table 1) and the accumulation of thylakoid proteins (Fig. 3A). We conclude that the mutation in At1g62640 leads to the pleiotropic defects including the drastic reduction of Fv/Fm after chilling treatment (Fig. 1).

At1g62640 encodes KasIII, which is involved in de novo synthesis of fatty acids in plastids (Fig. 4C). KasIII catalyzes decarboxy condensation of acetyl-CoA and malonyl-ACP, and the resulting 3-ketobutyryl-ACP is subjected to three steps of reaction, reduction, dehydration, and then reduction again, leading to the formation of a fully reduced form of butyryl-ACP. Butyryl-ACP acts as a primer for the sequential addition of malonyl groups, and the resulting primary products are palmitic acid (16:0) and stearic acid (18:0). Since de novo fatty acid synthesis is essential for plant growth, the amino acid alteration in KasIII is likely to cause a partial loss of enzymatic activity and consequent hypersensitivity of the photosynthetic machinery in thylakoid membranes to chilling stress.

Fatty acid content is reduced in kas3

We evaluated the kas3 phenotype by comparing the fatty acid content between the wild type and kas3 (Table 2). Before the chilling treatment, the level of total fatty acids (C16, C18 and C20) was reduced to 75% in kas3 compared with the wild type. Chilling treatment did not affect total fatty acid levels in the wild type (Table 2). However, total fatty acid levels were slightly more severely affected, being reduced to 60% in kas3 after chilling treatment compared with that in the wild type. This result suggests that de novo synthesis of fatty acids dependent on the full activity of KasIII is required for the maintenance of fatty acid levels during chilling treatment. The mutant phenotype indicates that this process is also required for stabilizing PSII, PSI and Cyt b₆f; and especially for maintaining PSII activity.

Table 1 shows comparison of the fatty acid composition between wild-type and kas3 plants. Both before and after the chilling treatment, the C16s/C18s ratios were higher in the wild type than in kas3 (Table 2). Since kas3 contains less total fatty acids than the wild type, the impact of this reduction was more severe on C16s than on C18s. After the shift to low temperature, the ratio of saturated fatty acids significantly increased in the wild type. The change was more apparent in C16s: the relative content of 16:0 increased from 17.9 to 24.8%
due to the temperature shift, while the 16:3 ratio decreased from 10.4 to 7.1% (Table 2). The relative content of 18:3 also decreased from 38.3 to 34.1% in the wild type. In kas3, a similar trend was observed, especially in C16s: the 16:0 ratio increased from 12.8 to 15.3% due to the temperature shift, while the 16:3 ratio decreased from 8.6 to 6.8% (Table 2). Notably, the ratio of 18:3 was higher in kas3 than in the wild type before the stress, and the ratio did not change after the temperature shift (Table 2).

Mechanism of PSII photodamage in kas3

kas3 has a partial defect in de novo fatty acid synthesis that results in reduced total fatty acid levels (Table 2) and also in a slight alteration in the ratio of unsaturated fatty acids (Table 2). Consistent with this primary defect, kas3 mutants show the pleiotropic phenotypes in their photosynthetic electron transport machinery at low temperature (Figs. 1–3). However, kas3 was isolated based on the hypersensitivity of PSII to low temperature (Fig. 1). The actual photoinhibition in vivo is a result of imbalance between the inactivation of D1 and its repair process. To assess which process is affected in kas3 at low temperatures, detached leaves of wild-type and kas3 plants were exposed to moderate light (300 μmol photons m⁻² s⁻¹) at 4°C and the resulting PSII photoinhibition was monitored as the decline in Fᵥ/Fₘ in the presence or absence of lincomycin. In the absence of lincomycin, Fᵥ/Fₘ declined more drastically in kas3 than in the wild type (Fig. 5A), which was consistent with the result in seedlings (Table 1). Lincomycin inhibits translation in chloroplasts, allowing the rate of PSII photodamage to be evaluated independently of the repair of damaged PSII. In the presence of lincomycin, the Fᵥ/Fₘ decline was faster in both kas3 and the wild type, but it was still more drastic in kas3 than in the wild type. The results indicate that PSII photodamage is more severe in kas3 than in the wild type, suggesting that the altered lipid environment makes PSII more sensitive to light.

Although PSII photodamage was faster in kas3 than in the wild type, it was still likely that PSII repair is also affected. To test this possibility, we monitored the repair process of damaged PSII as changes in Fᵥ/Fₘ after treatment (300 μmol photons m⁻² s⁻¹ at 4°C for 2 h) in both the wild type and kas3 in the absence of lincomycin (Fig. 5B). This treatment induced a 25% reduction from the initial Fᵥ/Fₘ level in kas3. In the wild type, the effect of the chilling treatment was much milder, and the Fᵥ/Fₘ level was recovered within 1 h (Fig. 5B). Although the Fᵥ/Fₘ level was slightly recovered in kas3 with slower kinetics (within 3 h) than in the wild type, the majority of PSII activity was irreversibly impaired. The longer chilling treatment induces slightly more severe PSII damage (Fᵥ/Fₘ = 0.65) in the wild type, but the PSII activity was also recovered within 1 h (data not shown). These results suggest that the altered lipid environment also affects the PSII repair cycle.

To characterize the kas3 defect in the PSII repair cycle, kas3 was crossed with two alleles of the var2 (yellow variegation 2)
mutant defective in the FtsH2 protease. FtsH2 is believed to be involved in the degradation of damaged D1 in the PSII repair cycle, and its defect partially abolishes chloroplast development, leading to leaf variegation (Sakamoto et al. 2003, Kato et al. 2007). Variegation is more severe in var2-1 than in var2-13 (Fig. 6A). Unexpectedly, the var2 variegation was partially suppressed in the double mutants (Fig. 6A). This result does not suggest that kas3 is impaired in the process relating to the removal of damaged D1. Based on the analogy with other var2 suppressors (see the Discussion for details), however, it is possible that the translation in chloroplasts is slower in kas3, resulting in the suppression of var2 variegation.

The effect of the kas3 defect on the Cyt b6f complex

In addition to PSII, the level of Cyt f was also reduced in kas3 after the chilling treatment. This result implies that the alterations in the lipid environments directly affect the stability and function of the Cyt b6f complex rather than being a secondary effect by PSII photoinhibition. To test this possibility, kas3 was crossed with the pgr1 (proton gradient regulation 1) mutant. pgr1 is conditionally defective in the activity of the Cyt b6f complex due to an amino acid alteration in the Rieske subunit (Munekage et al. 2001, Jahns et al. 2002). At low light intensities, the ETR was not affected in pgr1 as reported previously (Munekage et al. 2001), consistently with the nearly wild-type growth of pgr1 (Fig. 6B). In contrast, the kas3 pgr1 double mutant showed a drastic growth phenotype. The ETR was also more drastically affected in kas3 pgr1 than in the single mutants (Fig. 6C). However, there were no significant differences in the Cyt f levels among the wild type, kas3, pgr1 and kas3 pgr1 (Fig. 3B). The synergistic effect of kas3 and pgr1 suggests that the alterations in the lipid environment slightly affect the structure and/or activity of the Cyt b6f complex even at 23°C, even though PSII activity is not severely impaired. We conclude that the lipid environment is not optimized for the Cyt b6f complex in kas3, which affects the stability of the complex at low temperatures and its activity in kas3 pgr1 even at 23°C.

Discussion

Physiological function of de novo synthesis of fatty acids at low temperature

De novo fatty acid biosynthesis is essential for survival because it plays fundamental roles in producing basic cellular constituents including phospholipids and glycolipids. The phenotype of mutants defective in this pathway depends on how extensively the enzymatic activity is impaired. Despite the drastic reduction of enoyl-ACP reductase activity in the Arabidopsis mod1 (mosaic death 1) mutant (Fig. 4C), the total lipid content is only mildly affected (a 10% reduction) (Mou et al. 2000). However, the mutant exhibits pleiotropic phenotypes in plant growth and development and causes premature cell death. Based on the level of total fatty acids (25% reduction at 23°C),

Fig. 4 kas3 is defective in 3-ketoacyl ACP synthase III (KasIII). (A) The kas3 mutation was mapped to At1g62640. The kas3 mutation was mapped on the three bacterial artificial chromosomes (F24O1, T3P18 and F23N19) between two molecular markers on F24O1 and F23N19 (vertical bars) on chromosome 1. A nucleotide alteration (G to A) that changes Gly196 to serine and causing the kas3 phenotype was finally identified in the fifth exon of At1g62640. The red boxes indicate exons. (B) Alignment of KasIII protein sequences containing the region including the 196Gly affected in kas3. Spinach (Spinacia oleracea), rice (Oryza sativa), maize (Zea mays), Physcomitrella (Physcomitrella patens), Chlamydomonas (Chlamydomonas reinhardtii) and Synechosystis (Synechosystis sp. PCC6803). (C) De novo synthesis pathway of fatty acids in plastids. ACCase, acetyl-CoA carboxylase; ENR, enoyl-ACP reductase; ER, endoplasmic reticulum; KasI, KasII (At1g06), KasIII (At1g62640).
kas3 is likely to be more severely defective in fatty acid biosynthesis than mod1. However, kas3 does not show any phenotypes in development, and kas3 was isolated based on the sensitivity of PSII to low temperature (Fig. 1). During plant development, de novo fatty acid synthesis may be restricted at different enzymatic reactions, and consequently the developmental phenotype may not simply reflect the fatty acid levels in mature seedlings.

The Arabidopsis fabI mutant is defective in KasII activity (Fig. 4C) and was isolated as a mutant accumulating a high level of 16:0 (Wu et al. 1997). Since KasII is involved in the elongation of 16:0-ACP to 18:0-ACP, the lipid composition is rather different from that of kas3 that accumulates more C18s than the wild type. Although fabI is also sensitive to low temperature, it takes >7 d at 2°C to detect the drastic decline in Fv/Fm (Wu et al. 1997). This contrasts with the fact that PSII is severely inhibited at 4°C within 6 h in kas3 (Fig. 5), suggesting different mechanisms for the sensitivity to low temperature in the two mutants.

An increase in unsaturation of thylakoid lipids coincides with cold acclimation (Murata et al. 1992, Hugly and Somerville 1992, Routaboul et al. 2000), suggesting a physiological link. However, the level of 16:0 increased in the wild type after the temperature shift. Thus, it is unlikely that the hypersensitivity of PSII to low temperature is explained by the levels of unsaturated fatty acids in kas3. The fabI mutant of Synechocystis sp. PCC6803 is defective in enoyl-ACP reductase and sensitive to high temperature (Nanjo et al. 2010).

Defective fatty acid biosynthesis accelerates PSII photoinhibition

kas3 was isolated based on the hypersensitivity of PSII to low temperature. The Fv/Fm level was only slightly affected at 23°C in kas3 (Table 1), suggesting that full KasIII activity is required for the maintenance of PSII, especially during chilling treatment. To induce sufficient PSII photodamage in the experimental time range using detached leaves, we increased the light intensity to 300 µmol photons m⁻² s⁻¹, and PSII photodamage was observed even in the wild type at 4°C. For this technical reason, the

| Table 2 Fatty acid contents in the wild type and kas3 |
|-----------------|-----------------|-----------------|-----------------|
|                | 23°C Wild type  | 23°C kas3       | 4°C Wild type   | 4°C kas3        |
| Sum of C16s (%)| 34.9 ± 1.7      | 26.8 ± 1.5      | 36.5 ± 2.2      | 27.2 ± 1.4      |
| 16:0           | 17.9 ± 0.7      | 12.8 ± 0.4      | 24.8 ± 1.3      | 15.3 ± 0.8      |
| 16:1           | 5.3 ± 0.2       | 4.7 ± 0.1       | 3.7 ± 0.2       | 4.8 ± 0.2       |
| 16:2           | 1.3 ± 0.1       | 0.6 ± 0.2       | 0.9 ± 0.1       | 0.3 ± 0.1       |
| 16:3           | 10.4 ± 0.6      | 8.6 ± 0.7       | 7.1 ± 0.6       | 6.8 ± 0.4       |
| Sum of C18s (%)| 64.2 ± 1.7      | 72.4 ± 2.9      | 62.8 ± 3.0      | 71.8 ± 2.3      |
| 18:0           | 12.0 ± 1.0      | 2.5 ± 0.4       | 0.7 ± 0.1       | 2.1 ± 0.1       |
| 18:1           | 4.3 ± 0.2       | 2.4 ± 0.2       | 5.0 ± 0.2       | 0.7 ± 0.0       |
| 18:2           | 20.5 ± 0.6      | 22.2 ± 1.0      | 22.9 ± 0.9      | 20.9 ± 0.7      |
| 18:3           | 38.3 ± 0.8      | 45.2 ± 1.2      | 34.1 ± 1.9      | 48.1 ± 1.4      |
| Sum of C20 (%) | 0.8 ± 0.0       | 0.8 ± 0.3       | 0.7 ± 0.0       | 1.0 ± 0.0       |
| 20:0           | 0.8 ± 0.0       | 0.8 ± 0.3       | 0.7 ± 0.0       | 1.0 ± 0.0       |
| Total fatty acids (mg gFW⁻¹) | 3.46 ± 0.21 | 2.6 ± 0.32 | 3.51 ± 0.33 | 2.09 ± 0.09 |

Leaves were treated with 5% (v/v) HCl in methanol at 90°C for 1 h, and the samples were analyzed using gas chromatography. Fatty acid contents were compared before (23°C) and after chilling treatment (continuous light of 50 µmol photons m⁻² s⁻¹, 4°C for 7 d) in both the wild type and kas3. Means ± SD (n = 6).
mechanism of PSII photoinhibition is unlikely to be identical between the short time range (1–6 h) at 300 μmol photons m$^{-2}$ s$^{-1}$ and the longer time range (7 d) at 50 μmol photons m$^{-2}$ s$^{-1}$. However, we detected a more drastic decrease in $F_{v}/F_{m}$ at 4°C in both the presence and absence of lincomycin (Fig. 5A). This result indicates that PSII photodamage is accelerated at 4°C in the kas3 mutant, at least partly causing the kas3 phenotype in seedlings. Analysis of the pgsA mutant in Synechocystis sp. PCC6803, defective in biosynthesis of PG, indicated that the absence of PG also accelerated PSII photodamage, but it caused more drastic effects on the repair cycle of D1 by affecting the dimerization and reactivation of the PSII core complex (Sakurai et al. 2003). The kas3 defect is also likely to affect the repair of PSII, since the lipid environment may not be optimized for the repair cycle. This idea was supported by the results of phenotypic analysis of the double mutants with pgr1 and var2. (A) Phenotypes of the wild type (WT), kas3, var2-1, var2-13, kas3 var2-1 and kas3 var2-13. (B) WT, kas3, pgr1 and kas3 pgr1. All the seedlings were cultured at 23°C. (C) Light intensity dependence of the relative electron transport rate (ETR) in seedlings cultured at 23°C. The ETR was represented as relative values of the maximum ETR in the wild type (100%). The values are averages of five seedlings and the bars represent the SD.
monitoring the recovery process of damaged PSII (Fig. 5B). It is likely that the hypersensitivity of PSII to low temperature in \textit{kas3} is explained by both accelerated photodamage and an impaired PSII repair cycle.

We are not sure of the exact reason why PSII is more sensitive to low temperature in \textit{kas3}. In addition to the lower total fatty acid content, the composition of fatty acids was also slightly affected in \textit{kas3} (\textit{Table 2}). \textit{kas3} contains more 18:3 and less 16:0 than the wild type at 23°C (\textit{Table 2}), which may lead to more severe PSII photodamage at low temperatures. Inconsistent with this idea, a similar trend was observed in the wild type exposed to low temperature. The reduced de novo synthesis of fatty acids may mildly disturb PSII assembly, as detected by the slightly lower \( F_\text{v}/F_\text{m} \) (\textit{Table 1}) even at 23°C. It is also possible that the lipid environment surrounding PSII is not optimized. Although the problem is not serious for PSII at 23°C, it may cause the higher sensitivity of PSII to light at low temperature via a mechanism that does not occur in the wild type.

The \textit{var2} variegation phenotype is suppressed in the \textit{kas3} background

The \textit{var2} variegation was partially suppressed by introduction of the \textit{kas3} mutation (Fig. 6A). Recently, several \textit{var2} suppressors were identified including \textit{fug1} (\textit{fu-gaeri 1}) defective in \textit{cpIF2} (prokaryotic translation initiation factor 2) and \textit{scot} (\textit{snowy cotyledon 1}) defective in translation elongation factor G, both of which are involved in translation in chloroplasts (Miura et al. 2007). \textit{svr1} and \textit{svr2} (\textit{suppressor of variegation}) are defective in a chloroplast-localized homolog of pseudouridine synthase and ClpR1, respectively (Yu et al. 2008). Both mutants are also considered to be partially defective in chloroplast translation. Based on these observations, a balance between protein synthesis and degradation is hypothesized to be one of the factors determining the variegation phenotype (Miura et al. 2007, Yu et al. 2008). The \textit{kas3} defect may also partially impair translation in chloroplasts, resulting in the suppression of the \textit{var2} phenotype. It is also possible that the post-translational steps of D1 maturation are also partially affected in the \textit{kas3} mutant background. Consistent with this idea, the D1 level was lower than in the wild type even at 23°C, but the level of nucleus-encoded PsbO was only slightly affected even at 4°C (Fig. 3A). The results suggest that the minor reduction in the rate of translation in chloroplasts specifically affects the level of D1, which has a high turnover rate. It is possible that the full activity of KasIII is required for the rapid synthesis of the PSII reaction center proteins for the repair at low temperatures, as well as for protecting PSII from photodamage via unknown mechanisms.

The effect of the lipid environment on Cyt \( b_6f \) complex structure and activity

Consistent with the results of Chl fluorescence and P700 analysis (Fig. 2), protein blot analysis also suggests that electron transport is restricted at the Cyt \( b_6f \) complex, as well as PSII in \textit{kas3} at low temperature (Fig. 3A). Due to the single amino acid alteration (P194L) in the Rieske subunit of the Cyt \( b_6f \) complex (Munekage et al. 2001), the activity of the complex is hypersensitive to the low lumen pH that is achieved at high light intensity (Jahns et al. 2002). In the \textit{kas3 pgr1} double mutant, plant growth and ETR were severely impaired even at low light intensity (Fig. 6B, C). This result suggests that the alterations in the lipid environment surrounding the Cyt \( b_6f \) complex enhance the \textit{pgr1} phenotype even at low light intensities. This result also indicates that the full activity of KasIII is required even at 23°C, as evident in the low Chl content in \textit{kas3} (\textit{Table 1}). Consistent with the PSII phenotype, the level of Cyt \( f \) was drastically reduced at 4°C, suggesting the general function of de novo fatty acid synthesis at low temperature. It is also possible that a delay in de novo fatty acid synthesis influences the lipid composition of the thylakoid membranes, and a specific lipid factor may be necessary for the maintenance of the Cyt \( b_6f \) complex. In the \textit{Arabidopsis dgd1} mutant defective in DGDG accumulation, PSI activity is impaired by a limitation in the acceptor side (Ivanov et al. 2006). The function of each lipid component should be evaluated using mutants specifically defective in its biosynthesis.

Materials and Methods

Plant materials, growth conditions and chilling treatment

\textit{Arabidopsis thaliana} (ecotype Columbia \textit{gl1}) used in this work was grown in soil under growth chamber conditions (50 µmol photons m\(^{-2}\)s\(^{-1}\), 16 h photoperiod, 23°C). Three to four weeks after germination, plants were transferred to low temperature conditions (50 µmol photons m\(^{-2}\)s\(^{-1}\), continuous light, 4°C) and cultured further for 1 week (chilling treatment).

Chl measurement

Chl content was determined in fifth leaves using a Chl meter (SPAD-502, Konica Minolta, Tokyo, Japan) and was calculated as 0.65× SPAD value (µg cm\(^{-2}\)).

Chl fluorescence and P700 analysis

Chl fluorescence images were captured by a FluorCAM 700MF (PSI, Brno, Czech Republic). Chl fluorescence was measured using a MINI-PAM portable Chl fluorometer (Walz, Effeltrich, Germany). \( \Phi_{\text{PSII}} \) was calculated as \( (F_{\text{m}'} - F_i)/F_{\text{m}'} \), where \( F_{\text{m}'} \) is the maximum fluorescence level in the light, and \( F_i \) is the steady-state fluorescence level. ETR was calculated as \( \Phi_{\text{PSII}} \times \text{photon flux density (µmol photons m}^{-2}\text{s}^{-1}) \). NPQ was calculated as \( (F_{\text{m}} - F_{\text{m}'})/F_{\text{m}'} \). The redox change in P700 was assessed by monitoring absorbance at 810 nm using a PAM Chl fluorometer equipped with an emitter–detector unit ED P7000DW (Walz, Effeltrich, Germany) as described previously (Munekage et al. 2002).
Immunoblot analysis

For immunoblot analysis, thylakoid proteins were loaded onto an SDS–polyacrylamide gel on an equal Chl basis. The signals were detected using an ECL Plus Western Blotting Detection Kit (GE Healthcare UK Ltd, Buckinghamshire, UK), and visualized by an LAS3000 chemiluminescence analyzer (Fuji Film, Tokyo, Japan). The results presented are representative of three independent experiments.

Map-based cloning

The kas3 mutation was mapped using molecular markers based on cleaved amplified polymorphic sequences (Konieczny and Ausubel 1993). Genomic DNA was isolated from F₂ and F₃ plants derived from a cross between kas3 (genetic background of Columbia Gl1) and the wild type (Landsberg erecta). Homozygous F₁ and F₂ plants (kas3/kas3) exhibiting low Fᵥ/Fₘ levels after the chilling treatment were selected. At1g62640 was amplified by PCR using Ex Taq DNA polymerase (TAKARA, Kyoto, Japan) using the genomic DNA of the wild type and kas3. The PCR products were directly sequenced using a dye terminator cycle sequencing kit and an ABI prism3100 sequencer (Perkin-Elmer, Norwalk, CT, USA). The primer sequences for the f2401 marker are 5′-CTCTTGCCGAAGTACAGTGG-3′ and 5′-GGTGAGGATGTGTTGCGTC-3′, while those for the F23N19 marker are 5′-AACTAATATCAGCTTCATCTAG-3′ and 5′-GTTTCTTATATGATAACCATGC-3′. After PCR, the fragments were digested with EcoRI and MbolI, respectively.

For the complementation of kas3, the wild-type genomic sequence including At1g62640 was amplified (from 5′-CGAGAATTCCGATCCATCCT-3′ to 5′-ATAATCCCTTAAAGCTA AAC-3′). The PCR product was subcloned into pBIN19. The resulting plasmid was introduced into the Agrobacterium tumefaciens CS8C1 strain and the bacteria were used to transform homozygous kas3 plants by floral dipping (Clough and Bent 1998).

Fatty acid analysis

Detached leaves were treated with 5% (v/v) HCl in methanol and 40 µM pentadecanoic acid as an internal standard at 90°C for 1 h. The resulting methyl esters were dissolved in hexane and then analyzed using a gas chromatograph (GC-2014, Shimazu, Kyoto, Japan) equipped with a hydrogen-flame ionization detector. Fatty acid methyl esters were separated on a capillary column (ULBON HR-SS-10, 0.25 mm i.d.×25 m, Shinwaxhemo Co., Ltd, Kyoto, Japan). Fatty acid methyl esters were identified and quantified by comparing their peak positions and the peak areas with those of standards. We confirmed that there was no difference in fatty acid contents and compositions, when they were extracted by organic solvent and then used for methanalysis.

Analysis of PSII photoinhibition and recovery

PSII photoinhibition was monitored as the decline in Fᵥ/Fₘ in the presence or absence of lincomycin, an inhibitor of translation in plastids, at 300 µmol photons m⁻² s⁻¹ at 4°C. Detached leaves of the wild type and kas3 were incubated with 100 µg ml⁻¹ lincomycin for 30 min at room temperature and then placed under light at 4°C. The Fᵥ/Fₘ was measured after 1, 2, 3, 4 and 6 h treatments in the light. The recovery process of damaged PSII was also monitored as the changes in Fᵥ/Fₘ. Detached leaves of the wild type and kas3 were exposed to moderate light (300 µmol photons m⁻² s⁻¹) at 4°C for 2 h, and then transferred to 50 µmol photons m⁻² s⁻¹ at 23°C. The Fᵥ/Fₘ was measured after 1, 2, 3, 4, 5, 8 and 14 h treatments from starting the chilling treatment.

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